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harbored in pulmonary and surgical forms of tuberculosis, deduced by the injection of infected tissues, points out the fact that the number of bacilli introduced are of utmost importance, yet his own work only crudely took amounts into consideration.

Vagedes,³ who examined the bacilli from 30 cultures (6 of these from human sputum) by injecting them into rabbits fell into the same pitfall, as did also Lartigau,⁴ who believed he saw a definite relation between the virulence of the bacilli and the clinical course of the disease; chronic course associated with a low virulent bacillus and rapid course and miliary tuberculosis associated with high virulence of the bacilli. Veszpremi⁵ examined 10 cultures (2 of them laboratory strains and 8 recently isolated from cases of pulmonary tuberculosis) by intravenous injection into rabbits, using 4 rabbits for each culture, two receiving about $\frac{1}{2}$ mg. and the other two 3 mg. He concluded as a result of his observations that tubercle bacilli freshly isolated from cases of pulmonary tuberculosis reveal definite differences in virulence. Vagedes⁶ in a brief polemic criticizes Veszpremi's work because the latter obtained his cultures by passage through guinea-pigs before culture, and comments on the fact that their conclusions are alike with regard to the difference in virulence. Moeller⁷ studied a small number of cultures obtained from various types of tuberculosis and even though he believed he saw a difference in the virulence of these bacilli he saw no relation between the type of disease and the virulence for guinea-pigs. Dorset⁸ studied 12 cultures obtained by passage through guinea-pigs, 9 of these from human sources, of which 2 were from sputum and 3 from lungs direct. A rabbit and a guinea-pig were inoculated from each culture on egg medium and conclusions were drawn on the basis of the duration of life and macroscopic and microscopic findings after death. A suspension equal in density to a 24-hour typhoid culture was injected in 0.5 c c amounts. He concludes that there is a considerable variation in the virulence of different strains of human tubercle bacilli for rabbits and guinea-pigs. Fraenkel and Baumann⁹ obtained 37 cultures from human sources, 30 of these from the sputum were isolated with Heyden agar directly. Their emulsions

³ Ztschr. f. Hyg. u. Infektionskrankh., 1898, 28, p. 276.

⁴ Jour. Med. Research, 1901, 6, p. 156.

⁵ Centralbl. Bakt., I, O, 1903, 33, p. 176 and p. 255.

⁶ Ibid., p. 679.

⁷ Ztschr. f. Tuberk. u. Heilstättenw., 1903-4, 5, p. 5.

⁸ U. S. Dept. Agricult. 21st Ann. Report Bur. An. Ind., 1904, p. 159.

⁹ Ztschr. f. Hyg. u. Infektionskr., 1906, 54, p. 246, and 55, p. 327.

were made from 4-week old cultures which had been continued on coagulated glycerol cow serum. Their experience with rabbits, rats and mice led them to conclude that they were unsuitable for virulence tests, especially for revealing differences in pathogenic properties of the tubercle bacilli. Guinea-pigs were found, however, to be satisfactory. They claim that of some cultures even one bacillus would cause tuberculosis, but the proof of this was lacking. Their reasoning was by analogy with *B. prodigiosus*, it having been found that there is one bacillus in one 10,000 millionth to one 100,000 millionth of a mg. (their paper is not entirely clear on the amount referred to or used in their experiments). They noted no appreciable decrease of virulence of cultures grown on artificial mediums for 6 years and conclude that the range of virulence of their cultures lay between one 1,000 millionth and one 100,000 millionth of a mg. Marmorek,¹⁰ in 1906, called attention to the fact that as a criterion of the infectious power of tubercle bacilli, as a measure of their virulence, it is necessary to note the distribution of the anatomic lesions and only in a very insignificant manner consider the duration of life. If a series of test animals are inoculated in the same way and at the same time there is found after a definite time period, an equal amount of distribution of lesions in each animal. The difference in the anatomic lesions is the best gauge of the rapidity of the infection. In a brief summary contribution, Rodet and Delanoe¹¹ report on 26 cultures obtained from sputum by passage through guinea-pigs and rabbits. They do not give the method of inoculation of the cultures, but conclude that bacilli which were most virulent for rabbits were also most virulent for the guinea-pigs. They believe that they saw a relation between the virulence of the bacilli and the rapidity of the case, low virulence bacilli from chronic prolonged cases of tuberculosis. In a brief presentation, Meyer and Lewis¹² state that the cultural characteristics and virulence for small animals of 4 cultures of tubercle bacilli, 2 from extremely chronic cases and 2 from very acute cases presented no points of distinction. Gilbert and Gregg¹³ inoculated individual tubercle bacilli counted by the Barber method and found as a result of the examination of 13 freshly isolated cultures (mostly from sputum) less than 1 month old, that the minimum lethal number

¹⁰ Berl. klin. Wchnschr., 1906, 43, p. 328.

¹¹ Comp. rend. Acad. d. sc., 1908, 147, p. 500.

¹² Tr. Natl. Assn. Study and Prevention of Tuberculosis, 1913, 9, p. 332.

¹³ Med. Rec., 1915, 88, p. 208.

was approximately from 10 to 120 bacilli (less than 10 bacilli were not tested and the time interval allowed for the development of the infection in the guinea-pigs which were used as test animals was also not noted). The authors state that "In spite of the small numbers of cultures as yet studied it is certainly safe to assert that great differences in virulence occur in virulent cultures obtained from various cases. Whether cultures obtained from varied organs vary regularly in virulence remains to be determined."

Webb and Gilbert¹⁴ during the course of experiments on immunity in tuberculosis in which they gave injections of definite numbers of tubercle bacilli found that less than 10 virulent human tubercle bacilli could infect a child. They also injected 60 bacilli of a culture of which 35 bacilli would infect a guinea-pig, into 2 young guinea-pigs, 1 pregnant female, a nursing female and a normal male. The young pigs died in 2½ and 3 months after infection, while the remainder were killed after 5 months, the pregnant female revealing extensive tuberculosis, the nursing female no tuberculosis, and the male a moderate amount of tuberculosis. The authors conclude from these experiments that very young pigs are much more susceptible to tuberculosis than older pigs given definite small numbers of bacilli.

That death in tuberculosis is to a great extent attributable to accident is again well emphasized in a recent article by Krause.¹⁵

The incidence of infection with bovine tubercle bacilli in pulmonary tuberculosis as determined from the sputum is summarized up to 1916 in a contribution by Wang.¹⁶ Of a total of 998 cases, "the bacilli from 3 cultures were found to be of a mixed type, both 'human' and 'bovine,' and from 4 of a pure 'bovine' type."

Since there are a number of unsettled points which are of definite import in determining the virulence of tubercle bacilli, preliminary experiments were carried out to either prove these as unessential precautions or if possible find and institute the necessary ones.

In order to determine whether there is an appreciable difference in the reaction of guinea-pigs to tubercle bacilli at various ages the following experiment was performed:

Eight male guinea-pigs from the same stock were selected for use; 4 about 3 months old and 4 about 1 year old. They were all injected subcutaneously in the left lower quadrant of the abdomen with 0.3 cc of a good emulsion of a culture V of virulent human tubercle bacilli containing 0.000,001 mg. of

¹⁴ Jour. Am. Med. Assn., 1914, 63, p. 1098.

¹⁵ Am. Rev. of Tuberculosis, 1917, I, p. 65.

¹⁶ Lancet, 1916, 2, p. 417.

TABLE 1

THE ANATOMIC DISTRIBUTION OF TUBERCULOSIS IN MALE GUINEA-PIGS OF DIFFERENT AGES

Days Sectioned	Young Pigs	Mature Pigs
42	+++	++
62	+++	++
	+++	++++
	+++	++++

* Throughout this paper — designates no microscopic anatomical tuberculosis; + distinctly enlarged local and slightly enlarged retroperitoneal glands; ++ enlarged local and retroperitoneal glands and slight involvement of the spleen; +++ enlarged local and retroperitoneal glands, spleen markedly involved, the peritracheal glands enlarged and the lungs slightly involved; ++++ massive involvement of all the glands, the spleen, lungs and liver.

culture per c.c.* They were then kept under identical conditions of feeding, etc., during the entire experimental period. Two of each set were killed 42 days after infection and the other two after 62 days.

It is to be noted from the foregoing experiment that when small numbers of human tubercle bacilli are injected subcutaneously into male guinea-pigs of 3 months and 1 year old there is no marked difference in the amount of anatomic involvement obtained on section after 42 and 62 days.

TABLE 2

VIRULENCE OF HUMAN TUBERCLE BACILLI FROM CULTURES AT VARIOUS TIMES

Age of Cultures in Months	Injections in Mg.*	Cultures		
		1	2	3
1	0.1	++++†	++++	++++
	0.001	++++	+++	++++
	0.000,001	+++	++	+++
	0.000,000,01	+	—	+++
2	0.001	+++	+++	++++
	0.000,001	+++	+++	+++
	0.000,000,01	—	—	+++
3	0.001	++++	+++	++++
	0.000,001	+++	++	++
	0.000,000,01	+	—	++

* All the guinea-pigs were sectioned about 2 months after infection. One guinea-pig was used for each dilution.

† See previous table for value of these markings.

In order to determine whether there is an appreciable difference between the virulence of human tubercle bacilli, which had been isolated by Petroff's method,¹⁷ when kept in the incubator at 37-38 C. with due precautions against drying out, etc., at 1 and 3 months (satisfactory growths for virulence tests are in most cases only obtained after 1-2 months' incubation subsequent to inoculation of the sputum) the following experiment was performed:

* Organism V only produced tuberculosis of the local glands in amount of 0.000,000,01 mg. after 2 months.

¹⁷ Jour. Exper. Med., 1915, 21, p. 38.

Two cultures of human tubercle bacilli were injected subcutaneously into male guinea-pigs all about the same size and about 6 months old after the cultures were 1, 2 and 3 months old, in amounts as specified in Table 2.

That the virulence of the bacilli is not appreciably altered on a second seeding into a gentian violet egg medium tube (Petroff) is shown in Table 3.

TABLE 3
VIRULENCE OF HUMAN TUBERCLE BACILLI ON PRIMARY AND SECOND CULTURE ON
PETROFF'S MEDIUM *

—		Cultures	
		1 † (2856)	2 (204)
Primary	0.1	++++‡	++++
	0.001	++++	+++
	0.000,001	+++	+++
	0.000,000,01	++	++
Second	0.000,001	+++	+++
	0.000,000,01	+	++
	0.000,000,000,1	—	++

* Culture 1 was transferred to the second Petroff tube six months and Culture 2 four months after the primary culture was started.

† All the guinea-pigs were sectioned about 2 months after infection. One male guinea-pig about 6 months old was used for each dilution.

‡ See Table 1 for value of these markings.

A glance at Tables 2 and 3 reveals that virulent human tubercle bacilli on Petroff's medium do not appreciably alter their virulence for guinea-pigs whether tested after 1 or 3 months, provided no detrimental influences are introduced such as drying out, light, etc., and that a second culture on Petroff's medium produces bacilli of equal virulence for guinea-pigs to that of the primary.

In preparation of the cultures directly from the sputum by Petroff's method they are exposed to the action of an equal volume of 3% NaOH for from 20-30 minutes at 37 C. Soparker¹⁸ found that tubercle bacilli would still grow well after exposure to 2½% NaOH (an equal volume of 5%) for 1-2 hours at 37 C.; 3 or more hours' exposure caused a definite reduction in colonies while some even resisted destruction for as long as 24 hours. He did not, however, determine the effect of such treatment on the virulence of the bacilli, and for this purpose the following experiments were performed:

Three freshly isolated cultures of human tubercle bacilli were carefully emulsified and to 0.1 c.c. of various amounts, after reserving equal quantities in salt solution for control injections, were added 1 c.c. of 3% NaOH solution. These mixtures were all kept at 37 C. for 1 hour when they were neutralized,

¹⁸ Indian Jour. Med. Research, 1916, 4, p. 28.

carefully avoiding overheating, with hydrochloric acid using phenolphthalein as indicator, and then were injected into male guinea-pigs about 6 months old.

Two freshly isolated cultures of human tubercle bacilli were likewise used but treated with 6% NaOH for 1 hour.

The results of these 2 experiments are recorded in Table 4.

TABLE 4
THE EFFECT OF EXPOSURE TO 3 AND 6% NaOH FOR 1 HOUR ON THE VIRULENCE OF
HUMAN TUBERCLE BACILLI

Amount of Tubercle Bacilli Injected, in Mg.	Cultures									
	1		2		3		4		5	
	Control	3% NaOH	Control	3% NaOH	Control	3% NaOH	Control	6% NaOH	Control	6% NaOH
0.1	+++*	++	+++++	+++++	+++	+++	+++†	+	+++	+++
0.001	++	+++	+++	+++++	++	+++	++	—	+++	++
0.000,001	++	+	++	++	++	++	+++	—	+++	++
0.000,000,01	—	—	++	++	++	++	++	—	++	++

* The guinea-pigs were sectioned about 2 months after inoculation.

† It is noted in some cases that the larger doses of tubercle bacilli not infrequently result in less anatomic involvement than the smaller doses; this may be due to the fact that in the larger doses ulcers are frequently formed locally resulting in discharge of a considerable part of the tuberculous mass.

Since in the Petroff method only an equal volume of 3% NaOH solution is added to the sputum and incubation is only continued for 30 minutes, it is safe to conclude that this cannot materially affect the virulence of the bacilli in the original sputum. NaOH solution, 3%, at 37 C. for 1 hour has no appreciable effect on the virulence of human tubercle bacilli for guinea-pigs, while 6% may destroy the virulence of some cultures within 1 hour.

In order to determine whether there might be any difference in the virulence of the cultures of human tubercle bacilli isolated from the sputum of the same patient at different times by the method employed or in different cultures from the same sputum, the sputa from 4 cases were thus studied with the results given in Table 5.

From these experiments it is to be noted that the virulence of human tubercle bacilli isolated from the same sputum but grown on 2 different culture tubes do not differ appreciably within the limits of error of this method as tested in guinea-pigs. Likewise the bacilli isolated from the sputum of the same patient at different intervals (1 and 3 months) give concordant results (within the limit of error of the method) in virulence tests on guinea-pigs.

A series of preliminary experiments were performed in an attempt to note in a general way just how common a virulent or low virulent

TABLE 5
EXAMINATION OF DIFFERENT CULTURES ISOLATED FROM THE SAME SPUTUM AND FROM THE
SAME PATIENT AT DIFFERENT TIMES

Sputum		Results of Guinea-Pig Inoculations, Mg.			
		0.1	0.001	0.000,001	0.000,000,01
1	Initial 3 months later	+++ ++++	++ +++	++ +++	+ ++
2	Initial 1 month later	++++ +++	+++ +++	+ ++	— —
3	Both taken at same time	+++ ++++	lost +++	+++ +++	+ +
4	Both taken at same time	++++ ++++	++++ ++++	+++ +++	++ +++

tubercle bacilli could be isolated by culture from the sputum of consumptives positive by staining methods. Ten freshly isolated cultures were tested in dilutions as low as 0.001 mg. by subcutaneous injection, into guinea-pigs. Since not a single low virulent bacillus was found in this series it was decided to perform a large series of tests and carry the dilutions to a point at which only a small number of bacilli were present in the highest dilution. For this purpose the following technic was pursued throughout the remainder of the virulence tests:

The freshly isolated culture of tubercle bacilli on Petroff's medium (egg medium plus 1:10,000 Grüber's gentian violet from 1-2 months old) was weighed on a delicate balance weighing accurately to 0.1 mg. in a graduated 15 cc centrifuge tube and carefully emulsified, using a roughened glass rod for this purpose, first without and later gradually adding salt solution until there resulted an emulsion without any lumps (this requires about 20-30 minutes of careful grinding). This emulsion was then diluted so that 1 cc contained 1 mg., stirred well and allowed to stand 5 minutes when 1 cc drawn from the center of the emulsion was put into a second graduated centrifuge tube and diluted to 10 cc with sterile salt solution. Thus were made the following dilutions: 0.1, 0.001, 0.000,001, and 0.000,000,01 mg. contained in a cc. All the foregoing amounts were injected into male guinea-pigs about 4-7 months old, as soon as preparation was completed, and 0.1 mg. was injected intravenously into 1 or 2 mature rabbits to determine the type of bacillus, human or bovine. In order to get an approximation of the number of tubercle bacilli present in the above mentioned amounts, 10 of the 0.1 mg. dilutions were mixed as soon as prepared (after standing the tubercle bacilli would settle out and again become lumped so that simple mixing did not suffice in producing a good uniform emulsion) with an equal volume of oxalated blood of known red corpuscle content. This mixture was spread, as in making blood smears, on about a dozen slides, allowed to dry and fixed by methyl alcohol. The bacilli were then stained by the ordinary carbolfuchsin staining method either cold or warm. Usually a few good slides were obtained from each set and the proportion of tubercle bacilli to red cells noted. Various bloods were used

for this purpose but none possessed advantages over the others. The tubercle bacilli were usually found in small clumps of from 2-10 bacilli if the emulsion was well prepared.

The 10 emulsions counted revealed figures between 5.01 billion and 7.56 billion tubercle bacilli per mg. original culture. Thus the lowest dilution injected into the guinea-pigs contained approximately 50-75 tubercle bacilli. A total of 90 cultures from sputum were thus tested for their virulence within 1-2 (or in a few cases 3) months after isolation on Petroff's medium. The patients from whom the sputum was obtained for study of virulence ranged in age from 13-59 years, and were classified according to the National Association Classification into incipient, moderately advanced, and far advanced cases. As nearly as this could be approximated the duration of the disease and the rapidity of its course was considered.

TABLE 6
CLASSIFICATION OF CASES AND RESULTS OF ANIMAL INOCULATION OF CULTURES *

Classification†	Rabbit (Intravenously)					Guinea-Pig (Subcutaneously)											
	0.1 Mg.					0.000,001 Mg.								0.000,000,01 Mg.			
						Not Examined below this Amount				Examined below this Amount							
	-†	+	++	+++	++++	+	++	+++	+	++	+++	++++	+	++	+++	++++	
Incipient																	
Rapid course		1	1				1							1			
Slow course	1	3		2					1					4	1		
Moderately advanced																	
Rapid course	1	1		1					1				2				
Slow course	3	7	5	1	1	1			1	1		1	6	6	2		
Far advanced																	
Rapid course	9	10	1	5	2	1	1		1	1	1		14	7	1		
Slow course	4	10	9	3	5		2	4	1	2			8	5	7	2	
Totals (not including avirulent and bovine organisms).....	86					10			10				66				

* In a total of 90 cultures examined the following deviations from those tabulated were found:

One virulent organism which produced only local tuberculosis even in 1 mg. amount in the guinea-pig (none in the rabbit) obtained from a moderately advanced case running a very slow course.

One moderately virulent producing a + tuberculosis in the guinea-pig in 0.001 mg. amount (none in the rabbit) obtained from a far advanced case running a very rapid course.

One bovine organism from a far advanced case of very slow course which produced a + tuberculosis in the guinea-pig in 0.000,000,01 mg. amount.

One organism from a far advanced case with rapid course which produced a + tuberculosis in the guinea-pig in 0.000,000,01 mg. amount, and in 0.1 mg. amount intravenously in the rabbit produced after 2 months marked tuberculosis of the lungs, kidney and omentum, but not death.

† This classification is only an approximation as nearly as could be attained from the available clinical data on the cases.

‡ These markings indicate the amount of pulmonary involvement (the main, and generally only, organ affected macroscopically in the rabbits within 2 months after intravenous injection of 0.1 mg. human tubercle bacilli).

Reference to Table 6 and the appended notes reveals that of 90 cultures of tubercle bacilli isolated directly from the sputum of consumptives, 88 were of the human variety and, of these, 86 proved to

be virulent for guinea-pigs in 0.000,001 mg. amounts; 10 cultures were not examined below this amount and, of the remaining 76 that were examined in 0.000,000,01 mg. amounts, 66 produced definite metastatic tuberculosis within 2 months after inoculation. It is interesting to note that of the 2 low virulent organisms isolated from the sputum, 1 was absolutely innocuous to guinea-pigs even in 1 mg. amounts and was obtained from a moderately advanced case of very slow course which became a closed case under observation, and never presented appreciable constitutional symptoms. On the other hand, the other organism of low virulence which produced only a slight tuberculosis in guinea-pigs in 0.001 mg. amounts within 2 months was obtained from a far advanced case of very rapid course having passed from incipient to far advanced within 4 months and presenting severe constitutional symptoms until the performance of artificial pneumothorax. It is also to be noted that there does not seem to be any relation between the virulence of the tubercle bacilli for guinea-pigs or rabbits and the severity or rapidity of the disease in man. There seems to be just as little relation between the infection in rabbits and in guinea-pigs.

SUMMARY

The tubercle bacilli isolated by Petroff's method from the sputum of 90 cases of pulmonary tuberculosis in man, of which 8 were incipient, 20 moderately advanced and 62 far advanced, were found to be of the human variety in 88 of these cases. Of these 88 cultures examined for virulence to guinea-pig (by subcutaneous injection), 86 proved to be virulent in amounts of 0.000,001 mg., 10 of these were not examined below this amount and 66 produced tuberculosis (beyond the local glands) in guinea-pigs within 2 months in 0.000,000,01 mg. amounts. One of the 2 low virulent tubercle bacilli only produced a slight tuberculosis in guinea-pigs in 0.001 mg. amounts within 2 months, while the other produced no tuberculosis beyond the local involvement even in 1 mg. amounts.

No relation was observed between the virulence of the human tubercle bacilli for the guinea-pigs and rabbits, and the rapidity of the disease in man.

No appreciable difference, within the limits of error of the experiments, between the virulence of human tubercle bacilli tested by subcutaneous injection for young (4 months) and mature (1 year) male guinea-pigs was noted on section 42 and 62 days after infection.

Human tubercle bacilli isolated on Petroff's medium do not appreciably alter their virulence for a period of 1-3 months as tested in guinea-pigs provided no detrimental influences are introduced. A second seeding on Petroff's medium within this time produces bacilli of equal virulence to guinea-pigs to the primary.

Treatment of human tubercle bacilli with 3% NaOH solution for 1 hour at 37 C. does not appreciably affect the virulence of the bacilli for guinea-pigs while 6% NaOH for 1 hour at 37 C. will destroy the virulence of some cultures.

The test for virulence of human tubercle bacilli isolated from the sputum of the same patient at different intervals (1 and 3 months) give concordant results in guinea-pigs.